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Potential neuroprotective effects of acupuncture stimulation on diabetes mellitus in a global ischemic rat model

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Abstract

Acupuncture (ACU) is known to be effective in ischemia treatment, and glutamate (GLU) excitotoxicity is an important factor in neuronal cell death. We observed the effect of ACU on cerebral blood flow (%CBF) and ΔGLU (the changes in GLU release) in the ischemic stroke rat model of diabetic mellitus (DM). A global ischemia was induced using the eleven-vessel occlusion (11-VO) method in 14 Sprague-Dawley rats (DM), which were randomly divided into two groups: the control group and the ACU-treatment group. Extracellular ΔGLU was assessed using an intra-cerebral biosensor system measuring 256 samples per second, simultaneously with %CBF and electroencephalogram. ACU stimulation was applied to ACU points GB34 and GB39 during the ischemic period. Twenty-three diagnostic parameters were proposed first for a detailed analysis of changes in %CBF and GLU release during ischemia/reperfusion. ACU rats showed a significant decrease in ischemic ($p < 0.05$) and reperfusion %CBF ($p < 0.0001$) than control rats, and a significantly larger decrease in ischemic ΔGLU ($p < 0.05$) and peak level of reperfusion ΔGLU ($p < 0.005$) than control rats. From these results, we suggest that ACU stimulation is responsible for the potential protection

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of neurons through suppression of %CBF response in the increased plasma osmolality and extracellular ΔGLU in diabetic rats under ischemic conditions.

Keywords: acupuncture (ACU), diabetic mellitus (DM), ischemia, cerebral blood flow (CBF), glutamate (GLU), eleven-vessel occlusion (11-VO) model

Introduction

Acupuncture (ACU), an ancient art derived from East Asian medical tradition, has been one of the primary modalities applied to stroke patients in Korea. Reports on ACU in the medical literature have been anecdotal (Erickson 2000). Based on its widespread use, promising results, relatively fewer severe adverse effects, relatively low cost, and the insufficient quality of past research, a recent review recommended further research on ACU for stroke rehabilitation (Yang *et al* 2005).

Ischemic stroke, which comprises about 80% of all stroke cases, is one of the leading causes of death (Donnan *et al* 2008, Wu *et al* 2008). Under ischemic conditions, neuronal survival is responsible for the process of energy deprivation and resupply, and glutamate (GLU) excitotoxicity is the primary mechanism (Schuvailo *et al* 2006). Accumulated extracellular GLU aggravates and amplifies the impact of ischemic injury (Donnan *et al* 2008). Real-time *in vivo* monitoring of extracellular GLU release has been attempted using amperometric biosensor technology, with successful application in the field of medical research (Gardoni and Di Luca 2006, Siu *et al* 2004). Chuang *et al* (2007) reported that electro-ACU based on ACU stimulation using adopting stimulation instead of needle manual manipulation can attenuate GLU excess in ischemic injury in a common carotid artery occlusion (CAO) model on ACU points GV11 and GV16. On the other hand, Zhao and Cheng (1997) showed the effects of aspartate and taurin in ischemic injury in a middle cerebral artery occlusion (MCAO) model using electro-ACU on points GV8 and GV16.

Diabetes was reported to lead to an increase in morphologic brain damage and severity of neurologic deficits in animal models of global ischemia (Guyot *et al* 2000, 2001, Li *et al* 2000). In addition, pre-ischemic diabetic mellitus (DM) aggravates brain damage caused by transient global or forebrain ischemia (Muranyi *et al* 2006, Phillis *et al* 1999). Several studies have concluded that DM or hyperglycemia can be an independent risk factor for cerebrovascular events in diabetes. However, because the role of glucose concentration and neurological damage during brain ischemia in diabetes is not completely understood, the effects of DM on ischemia are controversial (Abarca and Bustos 1999, Zasslow *et al* 1989). In this study, we observed the effects of manual ACU on cerebral blood flow (CBF) and extracellular GLU concentration throughout induction of ischemic and reperfusion conditions in DM rats, using a 10 min eleven-vessel occlusion (11-VO) rat model. Changes in GLU release (ΔGLU) were monitored with electroencephalography (EEG) and two channels CBF.

Materials and methods

Animal preparation

Male Sprague-Dawley rats (200–250 g) were used in the experiment, and were housed in a 22–24 °C and 12 h light/dark cycle controlled environment. The animals were fed commercial rat chow and water *ad libitum*. Adequate injection and anesthesia were planned as a measure

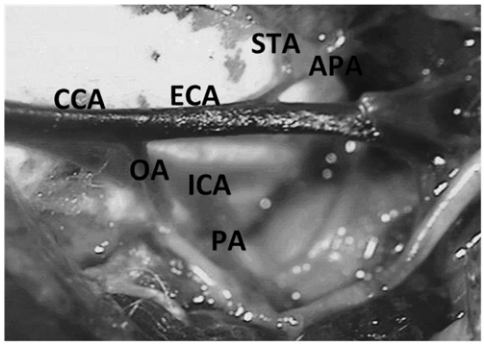

Figure 1. Vascular anatomy of neck dissection on the left side. APA = ascending pharyngeal artery; CCA = common carotid artery; ECA = external carotid artery; ICA = internal carotid artery; OA = occipital artery; PA = pterygopalatine artery; STA = superior thyroid artery.

to minimize pain or discomfort. All animal use procedures were approved by the Ethical Committee of the Kyung Hee University College of Medicine and were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Induction of diabetes mellitus (DM)

DM was induced by a single intraperitoneal injection of streptozocin (STZ, Sigma Chemical, St Louis, MO, USA), which was freshly dissolved in a 0.1 M citrate buffer at pH 4.5 and delivered at a dose of 60 mg kg^{-1} body weight. Blood glucose levels and body weights were determined 48 h after STZ injection. Animals with a blood glucose concentration >300 mg dL^{-1} were assigned to the DM group. Acute DM rats were used for experiments within five to seven days after STZ injection. There were two experimental groups; seven DM rats without ACU treatment (control group) and seven DM rats with ACU treatment (ACU group).

Surgical preparation

Animals were anesthetized with an intraperitoneal injection of 350 mg kg^{-1} chloral hydrate. Additional chloral hydrate was properly administered where needed in order to maintain the same level of anesthesia during the experiment. Rectal temperature was maintained regularly at 37.1 °C using a heating pad. An 11-VO was used as the global ischemia model (Caragine *et al* 1998). After dividing the omohyoid muscle, a pair of occipital arteries, the superior hypophyseal artery, ascending pharyngeal artery and pterygopalatine artery were coagulated with bipolar electrocautry (see figure 1). The ventral clivus just caudal to the basioccipital suture was then drilled and exposed to a 3 mm diameter. Next, the basilar artery was permanently coagulated.

Monitoring setup

Rats were placed in prone position using a stereotactic frame, and the skin was incised with a midline along the superior sagittal suture. Eight burr holes (figure 2) were made for real-time measurements of brain temperature, two channels EEG, GLU release, and two channels CBF. In detail, two burr holes were made 4 mm apart from the midline and 1 mm in front of the coronal suture for insertion of probes for GLU and brain temperature. At 2 mm intervals

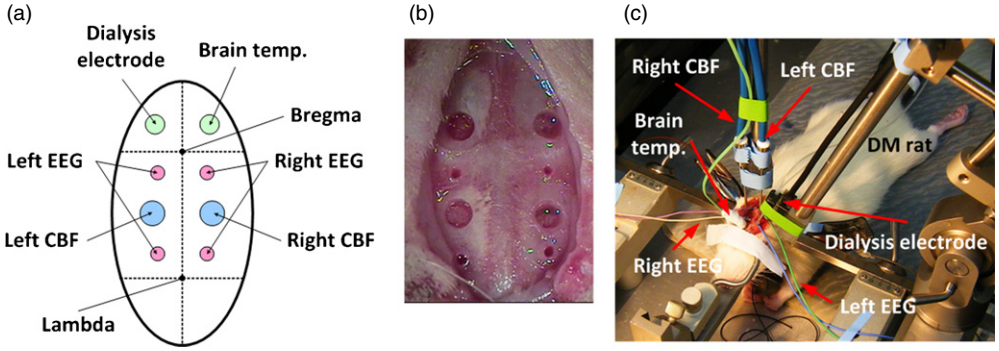

Figure 2. (a) Coordinates for craniotomy, (b) craniotomy in the rat, and (c) experimental setup for real-time monitoring of electroencephalography (EEG), cerebral blood flow (CBF), and extracellular glutamate (GLU) release during conditions of ischemia/reperfusion.

(This figure is in colour only in the electronic version)

from these holes, three burr holes were made on each side for two EEG probes and one CBF probe.

Four EEG electrodes were attached by screws and a reference electrode was attached to the skin of the right earlobe. EEG signals were pre-amplified using a 511 ac amplifier (Astro-Medical Inc., USA). These signals were used to examine electrical failure of neuronal cells. %CBF was measured using a BLF21D laser Doppler flow meter (Transonic Systems Inc., USA) with laser Doppler monofiber flowprobes (Type N, 18 gauge needle). Changes in GLU release were examined using a conventional dialysis electrode purchased from Sycopel International Ltd. The general 20-10-4-4 type dialysis electrode was filled with phosphate buffered saline (PBS) to electropolymerize the O-phenylenediamine on the platinum electrode at 0.65 V for 20 min using a Sycopel BD2000 potentiostat (Sycopel Int. Ltd, UK). The dialysis electrode was then perfused with fresh PBS containing GLU oxidase at a flow rate of 0.5 μL min^{-1}. The dialysis electrode showed a linear response in the concentration range of a standard GLU solution (50–450 μM), with a sensitivity of 0.23 nA μM^{-1}. The correlation coefficient for linear regression was $R^2 = 0.999$. Following the sensor calibration procedure, the micro-dialysis electrode was inserted into the motor cortex at coordinates A 1: L 4 : V 4 mm (from the bregma and the dura) through a small incision in the dura.

Induction of ischemia and reperfusion

A snare made with a rubber tube was hung from both common carotid arteries (CCAs), see figure 1. Using the 11-VO rat model, we estimated normal control data for 10 min; the snare was then retracted using gravity retraction with a 10–12 g weight. We confined the induction of ischemia in order to decrease %CBF and EEG amplitude compared to the base. Ischemia was induced for 10 min and then reperfused for 40 min.

Acupuncture stimulation

In the ACU group, ACU stimulation was applied throughout the ischemic period. Four ACU needles with a length of 40 mm and a diameter of 0.25 mm (Dongbang Acupuncture Inc., Republic of Korea) were manually inserted about 5 mm deep into ACU points just anterior and inferior to the fibular head (GB34, Yang Tomb Spring) and distal 4/5 sites on the imaginary

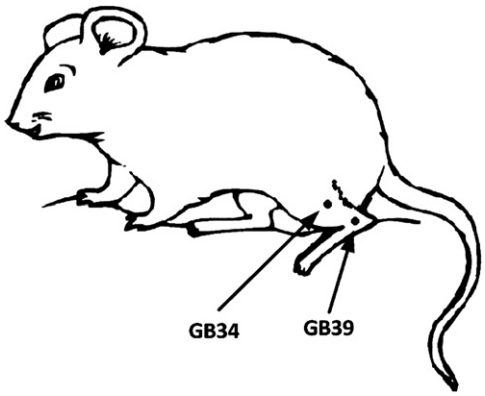

Figure 3. Two acupuncture points, GB34 (Yang Tomb Spring) and GB39 (Suspended Bell), were used in this study. GB34 is located just anterior and inferior to the fibular head. GB39 is located distal $4/5$ points on the imaginary line connecting the lateral side of the knee and the lateral malleolus of the tibiofibula.

line connecting the lateral side of the knee and the lateral malleolus of the tibiofibula (GB39, Suspended Bell), as shown in figure 3 (Yin *et al* 2008b). The needles were slowly rotated at a couple of alternate rotations of 180° s^{-1}, in such a fashion that one needle is rotated for 10 s, and the next needle is then rotated, while other needles remain inserted.

Analysis and diagnostic parameters

Waveforms obtained from laser Doppler flowmetry, EEG, and potentiostat for ΔGLU were displayed using the DASYLab data acquisition system (National Instruments, USA) and were analyzed with both Matlab 6.5 (The MathWorks Inc., USA) and DADiSP 2002 Evaluation Version B18 (DSP development Co., USA). According to reports found in the literature (Caragine *et al* 1998, Choi *et al* 2008, Kohno *et al* 1998, Lee *et al* 2009, Sugahara *et al* 2001, Yin *et al* 2008a, Zhao *et al* 1997, Zilkha *et al* 1995), we set up the basic waveforms for changes in %CBF and GLU release during conditions of ischemia and reperfusion, as shown in figure 4. From figure 4, we proposed 23 diagnostic parameters for a detailed analysis of changes in %CBF and GLU release during conditions of ischemia/reperfusion. Of these 23 diagnostic parameters, 11 were defined for analysis of changes in %CBF levels, and 13 were defined for analysis of changes in GLU release; one parameter T_{pri} was used in both waveforms. The definitions of these parameters are summarized in tables 1 and 2. Additionally, the slope information, including two CBF-related slopes, equations (1) and (2), and three ΔGLU-related slopes, equations (3) to (5), is given as follows:

$$S_{\text{Ci-dec}} = (100\% - \%\text{CBF}_i)/T_{i\text{-fall}}, \tag{1}$$

$$S_{\text{Crep-inc}} = (\%\text{CBF}_{\text{rep-max}} - \%\text{CBF}_i)/T_{\text{rep-rise}}, \tag{2}$$

$$S_{\text{Gi-inc}} = (\Delta\text{GLU}_{i\text{-max}} - \Delta\text{GLU}_{\text{pri-base}})/T_{i\text{-rise}}, \tag{3}$$

$$S_{\text{Grep-inc}} = (\Delta\text{GLU}_{\text{rep-max}} - \Delta\text{GLU}_{\text{pri-base}})/(T_{i\text{-rise}} + T_{\text{rep-delay}}), \tag{4}$$

$$S_{\text{Gi-dec}} = (\Delta\text{GLU}_{\text{pri-base}} - \Delta\text{GLU}_{\text{rep-max}})/T_{\text{rep-fall}}. \tag{5}$$

Table 1. Definition of parameters for analysis of changes in cerebral blood flow (CBF).

Parameter	Description
$\%CBF_{zero}$ (%)	Zero level of %CBF
$\%CBF_{pri\text{-}base}$ (%)	Base level of pre-ischemic %CBF
$\%CBF_{i}$ (%)	Ischemic %CBF level
$\%CBF_{rep\text{-}max}$ (%)	Maximum level of reperfusion %CBF
T_{pri} (s)	Time of pre-ischemia induction
$T_{i\text{-}fall}$ (s)	Time elapsed to real ischemia induction after manipulation
T_{i} (s)	Time of ischemia induction
$T_{rep\text{-}rise}$ (s)	Time elapsed to reach 5% of $\%CBF_{rep\text{-}max}$ after the onset of reperfusion period
$T_{rep\text{-}fall+base}$ (s)	Reperfusion time – $T_{rep\text{-}rise}$
$S_{Ci\text{-}dec}$ (% s^{-1})	Decrement slope in ischemic period, equation (1)
$S_{Crep\text{-}inc}$ (% s^{-1})	Increment slope in reperfusion period, equation (2)

Table 2. Definition of parameters for analysis of changes in extracellular glutamate (GLU) release.

Parameter	Description
ΔGLU_{zero} (μM)	Zero level of GLU release
$\Delta GLU_{pri\text{-}base}$ (μM)	Base level of pre-ischemic GLU release
$\Delta GLU_{i\text{-}max}$ (μM)	Maximum level of ischemic GLU release
$\Delta GLU_{rep\text{-}max}$ (μM)	Maximum level of reperfusion GLU release
T_{pri} (s)	Time of pre-ischemia induction
$T_{i\text{-}release}$ (s)	Time delay of the beginning of GLU release after the onset of ischemia
$T_{i\text{-}rise}$ (s)	Time elapsed to reach $\Delta GLU_{i\text{-}max}$ after the beginning of GLU release
$T_{rep\text{-}delay}$ (s)	Time elapsed to reach $\Delta GLU_{rep\text{-}max}$ after the onset of reperfusion period
$T_{rep\text{-}fall}$ (s)	Time elapsed to return to $\Delta GLU_{rep\text{-}base}$ in reperfusion period
$T_{rep\text{-}base}$ (s)	Time of $\Delta GLU_{rep\text{-}base}$ in reperfusion period
$S_{Gi\text{-}inc}$ (μM s^{-1})	Increment slope in ischemic period, equation (3)
$S_{Grep\text{-}inc}$ (μM s^{-1})	Increment slope in reperfusion period, equation (4)
$S_{Grep\text{-}dec}$ (μM s^{-1})	Decrement slope in reperfusion period, equation (5)

Statistics

Statistical analysis was performed to compare the mean changes in %CBF and GLU release between control and ACU groups using the two-tailed Student's *t*-test. *P*-values less than 0.05 were regarded as statistically significant.

Results

Streptozocin-induced DM rats

Blood glucose concentration was approximately three times higher at 48 h after STZ injection than before STZ injection (figure 5). Mean blood glucose levels before and 48 h after STZ injection were 111.75 $\pm$ 20.10 mg dL^{-1} and 392.75 $\pm$ 48.21 mg dL^{-1} ($p < 0.0001$), respectively, in control rats. Mean blood glucose levels before and 48 h after STZ injection were 132.14 $\pm$ 25.03 mg dL^{-1} and 361.00 $\pm$ 39.97 mg dL^{-1} ($p < 0.0001$), respectively, in ACU rats.

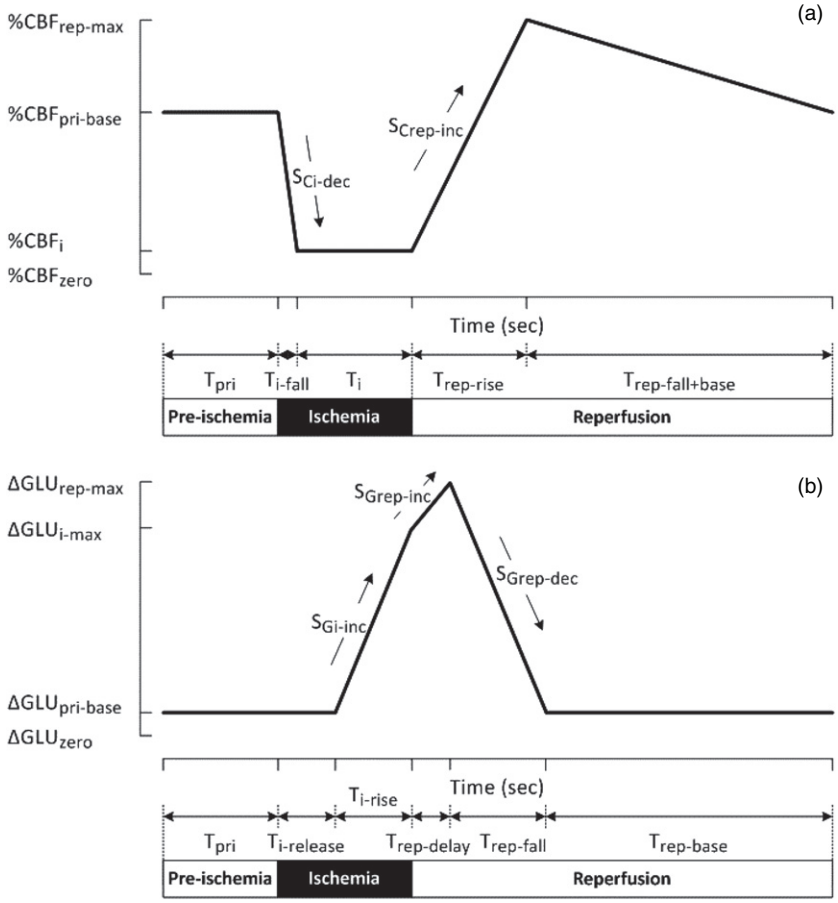

Figure 4. Definition of diagnostic parameters proposed for estimation changes in %CBF (a) and ΔGLU (b) throughout the experimental protocol: 10 min pre-ischemic (control) period, 10 min ischemic period, and 40 min reperfusion period. In figure (a), 11 parameters were defined for analysis of changes in %CBF during conditions of ischemia/reperfusion; 2 parameters, including $\%CBF_{pri\text{-}base}$ and T_{pri}, during the pre-ischemic period, 4 parameters, including $\%CBF_i$, $T_{i\text{-}fall}$, T_i and $S_{Ci\text{-}dec}$, during the ischemic period, and 4 parameters, including $\%CBF_{rep\text{-}max}$, $T_{rep\text{-}rise}$, $T_{rep\text{-}fall+base}$ and $S_{Crep\text{-}inc}$, during the reperfusion period. $\%CBF_{zero}$ was used to calculate all %CBF-related parameters. In figure (b), 13 parameters were defined for analysis of changes in GLU release during conditions of ischemia/reperfusion; 2 parameters, including $\Delta GLU_{pri\text{-}base}$ and T_{pri}, during the pre-ischemic period, 4 parameters, including $\Delta GLU_{i\text{-}max}$, $T_{i\text{-}release}$, $T_{i\text{-}rise}$ and $S_{Gi\text{-}inc}$, during the ischemic period, and 6 parameters, including $\Delta GLU_{rep\text{-}max}$, $T_{rep\text{-}delay}$, $T_{rep\text{-}fall}$, $T_{rep\text{-}base}$, $S_{Grep\text{-}inc}$ and $S_{Grep\text{-}dec}$, during the reperfusion period. ΔGLU_{zero} was used to calculate all ΔGLU-related parameters.

Changes in %cerebral blood flow response

Figures 6 and 7 show representative waveforms with respect to changes in %CBF (figure 6) and extracellular GLU release (figure 7) during an 11-VO model ischemia/reperfusion.

As shown in figure 6, the change pattern of ischemia-evoked %CBF responses in the 11-VO model of DM rats with ACU treatment was similar to that without ACU treatment. Mean time intervals ($S_{Ci\text{-}dec}$) between induction of ischemia and the beginning of the %CBF

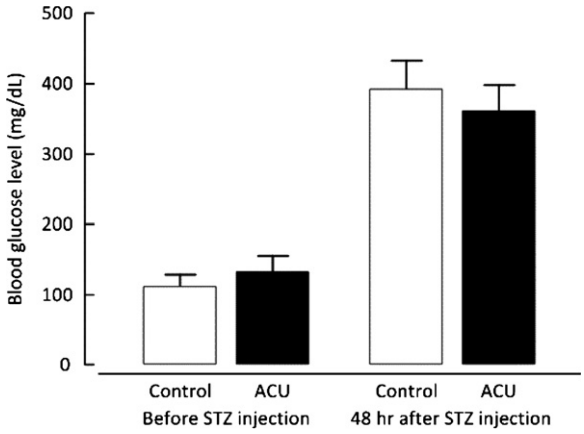

Figure 5. Blood glucose concentration before streptozocin injection and at 48 h after streptozocin injection. Each result is expressed as mean ± standard deviation of seven rats.

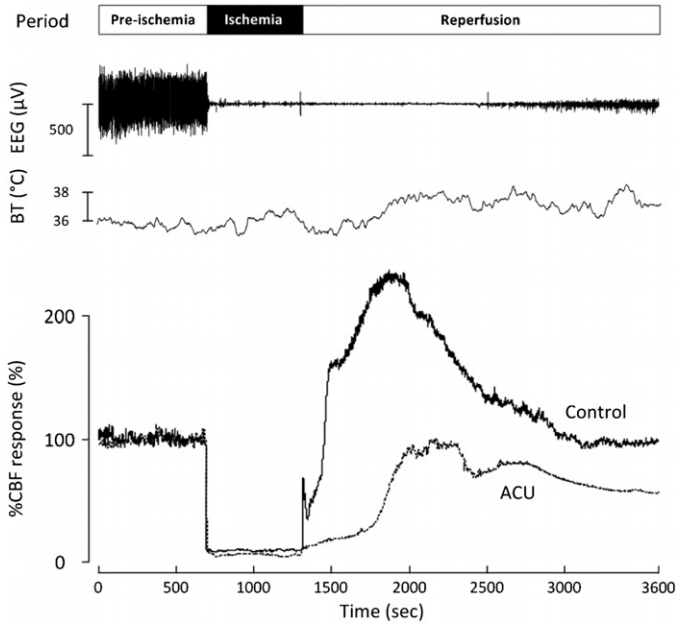

Figure 6. Representative real-time changes in ischemia-evoked EEG, brain temperature (BT) and %CBF during use of an eleven-vessel occlusion model in the control and ACU groups, measured using a laser Doppler flow meter.

plateau in the control group (13.26 ± 3.12 s) were similar to those in the ACU group (12.35 ± 2.97 s). After onset of ischemia, %CBF decreased rapidly to near 10% of original levels, and the EEG response became flat. These changes were maintained throughout the 10 min ischemic period. Decreases in ischemic %CBF (%CBF$_i$) were 11.10 ± 2.91% and 7.71 ± 2.55% for the control and ACU groups, respectively. After reperfusion in the controls, %CBF increased considerably, and then returned to the level of the baseline, along with an

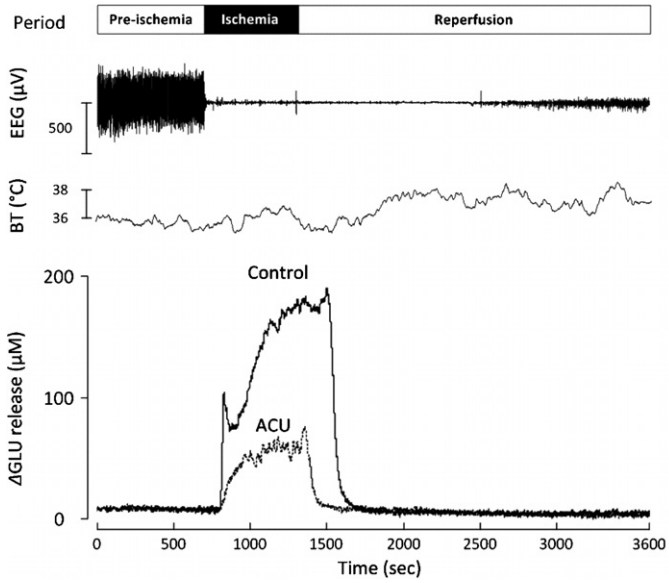

Figure 7. Representative real-time changes in ischemia-evoked EEG, BT and extracellular ΔGLU during use of an eleven-vessel occlusion model in the control and ACU groups, measured using a micro-dialysis electrode.

accompanying EEG recovery. ACU-treated rats showed patterns similar to those without ACU treatment. However, the maximum reperfusion %CBF response ($\%CBF_{rep\text{-}max}$) in the control was approximately three times that of the ACU group ($p < 0.0001$, $n = 7$). Also, the time elapsed to reach the maximum %CBF level ($T_{rep\text{-}rise}$) showed a significant increase ($p < 0.05$, $n = 7$) in DM rats with ACU treatment, 763.46 ± 173.28 s, compared to DM rats without ACU treatment, 994.96 ± 237.39 s. Thus, the increment slope during the reperfusion period ($S_{Crep\text{-}inc}$) associated with $\%CBF_{rep\text{-}max}$ and $T_{rep\text{-}rise}$ also showed a significant decrease in the ACU group compared to the control group ($p < 0.0001$, $n = 7$). As a result, the five parameters, including one ($\%CBF_i$) during the ischemic period and four ($\%CBF_{rep\text{-}max}$, $T_{rep\text{-}rise}$, $T_{rep\text{-}fall+base}$, and $S_{Crep\text{-}inc}$) during the reperfusion period, among the proposed diagnostic parameters of the ischemia-evoked %CBF response showed a significant difference between the two groups. Quantitative results are summarized in table 3.

Changes in extracellular glutamate release

As shown in figure 7, the change pattern of ischemia-evoked ΔGLU in an 11-VO model of DM rats with ACU treatment was also similar to that without ACU treatment. The mean time delay at the beginning of ΔGLU after onset of ischemia ($T_{i\text{-}release}$) in the control group (96.14 ± 22.56 s) was similar to that in the ACU group (90.40 ± 15.95 s). In both groups, rapid elevation of ΔGLU began at $T_{i\text{-}release}$ after onset of ischemia, and continued to rise throughout the 10 min ischemic period. Following reperfusion, ΔGLU showed a considerable decrease, and was then restored to the level of the pre-ischemic period at $T_{rep\text{-}delay} + T_{rep\text{-}fall}$ after reperfusion, along with accompanying EEG recovery. The time elapsed to reach $\Delta GLU_{rep\text{-}max}$ after onset of the reperfusion period ($T_{rep\text{-}delay}$) and the time elapsed to return to $\Delta GLU_{rep\text{-}base}$ during the reperfusion period ($T_{rep\text{-}fall}$) were not significant in either group. However, elevation

Table 3. Changes in ischemia-evoked %CBF levels and corresponding parameters in control and acupuncture groups.

Parameter	Control group	Acupuncture group	*P*-value
$\%CBF_{zero}$ (%)	0	0	
$\%CBF_{pri\text{-}base}$ (%)	100.00 ± 6.90	100.00 ± 11.90	N.S.[a]
$\%CBF_{i}$ (%)	11.10 ± 2.91	7.71 ± 2.55	=0.0120
$\%CBF_{rep\text{-}max}$ (%)	211 ± 58.45	71.88 ± 34.41	<0.0001
T_{pri} (s)	635.57 ± 29.75	617.29 ± 20.25	N.S.[a]
$T_{i\text{-}fall}$ (s)	6.85 ± 1.46	7.88 ± 2.10	N.S.[a]
T_{i} (s)	620.69 ± 17.00	615.88 ± 8.37	N.S.[a]
$T_{rep\text{-}rise}$ (sec)	763.46 ± 173.28	994.96 ± 237.39	=0.0180
$T_{rep\text{-}fall+base}$ (s)	1636.54 ± 173.28	1405.04 ± 237.39	=0.0180
$S_{Ci\text{-}dec}$ (% s^{-1})	13.26 ± 3.12	12.35 ± 2.97	N.S.[a]
$S_{Crep\text{-}inc}$ (% s^{-1})	0.32 ± 0.09	0.07 ± 0.04	<0.0001

[a] N.S. = Not significant.

of GLU release was suppressed in the ACU group, resulting in a decrease of approximately 70%. Maximum changes in extracellular ΔGLU during the ischemic period ($\Delta GLU_{i\text{-}max}$) were 182.24 ± 73.91 μM in the control group and 56.23 ± 13.93 μM in the ACU group ($p < 0.05$, $n = 7$), while those of extracellular GLU release during the reperfusion period ($\Delta GLU_{rep\text{-}max}$) were 196.70 ± 77.58 μM in the control group and 65.92 ± 19.40 μM in the ACU group ($p < 0.005$, $n = 7$). Corresponding slopes for equations (3)–(5) showed a significant decrease ($p < 0.05$, $n = 7$) in the ACU group: a 77.60% decrease in $S_{Gi\text{-}inc}$ ($p < 0.05$, $n = 7$), a 72.18% decrease in $S_{Grep\text{-}inc}$ ($p = 0.0164$, $n = 7$), and a 74.68% decrease in $S_{Grep\text{-}dec}$ ($p = 0.001$, $n = 7$), compared to the control group. As a result, the five parameters, including two ($\Delta GLU_{i\text{-}max}$ and $S_{Gi\text{-}inc}$) during the ischemic period and three ($\Delta GLU_{rep\text{-}max}$, $S_{Grep\text{-}inc}$ and $S_{Grep\text{-}dec}$) during the reperfusion period, among the proposed diagnostic parameters of ischemia-evoked ΔGLU showed a significant difference between the two groups. Quantitative results are summarized in table 4.

Discussion

Using a real-time *in vivo* monitoring method, this study examined the effects of ACU stimulation on %CBF and ΔGLU under devastating acute ischemic conditions in the 11-VO global ischemic model of DM rats. Patterns of %CBF response were consistent with those reported previously using the laser Doppler method, and patterns of ΔGLU were also consistent with those reported previously using the micro-dialysis electrode method (Caragine *et al* 1998, Choi *et al* 2008, Sugahara *et al* 2001, Lee *et al* 2009, Yin *et al* 2008a).

Diabetic stroke aggravated neurologic damage in both focal and global animal models (Ginsberg *et al* 1980, Li and Siesjo 1997, Pulsinelli *et al* 1982), and a similar effect was reported in diabetic stroke of humans (Longstreth and Inui 1984, Toni *et al* 1992). The correlation between DM and a poorer outcome after stroke has been examined in both human and animal studies. DM enhances cerebral ischemic injury, but whether or not it is directly related to the presence of diabetes is unclear.

ACU is generally known as an effective adjunctive therapy for stroke rehabilitation (Erickson 2000, Hsieh *et al* 2007). Previous studies have reported that ACU stimulation helps neuronal survival (Chuang *et al* 2007), plasticity (Ren *et al* 2008), and improvement of

Table 4. Changes in ischemia-evoked GLU levels and corresponding parameters in control and acupuncture groups.

Parameter	Control group	Acupuncture group	*P*-value
ΔGLU_{zero} (μM)	0	0	
$\Delta GLU_{pri\text{-}base}$ (μM)	2.32 ± 1.81	1.15 ± 0.44	N.S.[a]
$\Delta GLU_{i\text{-}max}$ (μM)	182.24 ± 73.91	56.23 ± 13.93	=0.0148
$\Delta GLU_{rep\text{-}max}$ (μM)	196.70 ± 77.58	65.92 ± 19.40	=0.0035
T_{pri} (s)	635.57 ± 29.75	617.29 ± 20.25	N.S.[a]
$T_{i\text{-}release}$ (s)	96.14 ± 22.56	90.40 ± 15.95	N.S.[a]
$T_{i\text{-}rise}$ (s)	532.43 ± 32.10	536.31 ± 11.82	N.S.[a]
$T_{rep\text{-}delay}$ (s)	44.78 ± 45.08	45.68 ± 44.79	N.S.[a]
$T_{rep\text{-}fall}$ (s)	225.59 ± 78.50	283.97 ± 52.11	N.S.[a]
$T_{rep\text{-}base}$ (s)	2129.94 ± 107.26	2070.35 ± 78.10	N.S.[a]
$S_{Gi\text{-}inc}$ (μM s^{-1})	46.92 ± 33.57	10.51 ± 2.47	=0.0287
$S_{Grep\text{-}inc}$ (μM s^{-1})	43.64 ± 25.02	12.14 ± 3.31	=0.0164
$S_{Grep\text{-}dec}$ (μM s^{-1})	81.67 ± 28.26	20.68 ± 8.86	=0.0010

[a] N.S. = Not significant.

spasticity in stroke conditions (Zhao *et al* 2009). However, the effects of ACU stimulation are controversial, and strong evidence remains to be discovered (Hopwood *et al* 2008, Wu *et al* 2008). These effects might be possible through modulation of GLU excitotoxicity and reperfusion hyperemia (Pang *et al* 2003), reduced lipid peroxidation (Siu *et al* 2004), enhanced dopamine level (Chuang *et al* 2007), facilitated neurogenesis (Yang *et al* 2005), and increased blood flow (Hsieh *et al* 2006). Therefore, the short-term effects of functional responses to ischemia-evoked %CBF and ΔGLU in DM rats with and without ACU treatment were examined using a real-time *in vivo* monitoring method.

Eleven-vessel occlusion (11-VO) model

We first observed the effect of ACU under conditions that were more acute and severe than in previously reported models, such as the MCAO (Zhao *et al* 1997) and CAO models (Pang *et al* 2003). The 11-VO global ischemic model used in this study has been well established as a severe global ischemia (Caragine *et al* 1998). Furthermore, we tried to find an ischemic model that yields reproducible and accurate results. The four-vessel occlusion (4-VO) is the most commonly used model for induced global ischemia. However, a disadvantage of the 4-VO is that it can induce an error in blocking of posterior collateral circulation for anatomical reasons (Caragine *et al* 1998, Pulsinelli and Buchan 1988). Generally, the alar foramen at the upper end of the C1 vertebral body is used to block posterior circulation supplied by the vertebral artery. The hole is anatomically small, and identification of a clear occlusion of the collateral blood flow, caused by the burst of venous plexus before coagulation of the artery, is difficult. Therefore, we used an 11-VO model where collateral blood flow could be occluded, compared to the 4-VO model. We performed surgery on the occlusion of blood flow by blocking the basilar artery, instead of the vertebral arteries and anterior spinal artery. Therefore, results showed that use of the 11-VO model led to an immediate drop in %CBF to near zero levels and maximized ΔGLU while maintaining self-respiration to mimic physiological forebrain ischemia, compared to the traditional 4-VO model.

Changes in %cerebral blood flow response

In regard to %CBF response, results showed that a decrease in %CBF to near 10% of the baseline level occurred after completion of 11-VO, which was maintained during the 10 min ischemic period. A criterion of a >70% decrease in %CBF on the occluded side is frequently used to determine the success of the occlusion (Chen *et al* 2008, Newcomb *et al* 2006, Scholler *et al* 2004). These results showed $a > 89\%$ decrease in %CBF in both groups. In particular, ACU rats showed a significantly larger decrease ($p < 0.05$, $n = 7$) in %CBF (3.39%) during ischemia than control rats. Therefore, surgical and occlusion setups, supported by reduced %CBF in occlusion, are well established, and a real-time method is very useful for monitoring blood flow in the cerebral space. During the reperfusion period, results showed that a significant decrease ($p < 0.0001$, $n = 7$) in peak changes in %CBF was observed in the ACU group compared to the control.

Commonly, %CBF increases after onset of ischemia, and considerable reactive oxygen is produced during the reperfusion period as a result of increased oxygen supply and metabolism, or reperfusion injury (Aronowski *et al* 1997, Leff and Repine 1990, Strasser *et al* 1994). Some studies (Ginsberg *et al* 1980, Kawai *et al* 1998, Wagner *et al* 1992) have shown that diabetic stroke might be associated with a decrease in reperfusion %CBF. Although there may be negative reports showing no significant neuroprotective effects, ACU stimulation in diabetic stroke might be responsible for cellular protective effects and improvement in CBF (Domenici *et al* 2003, Yin *et al* 2008a). Zhao *et al* (2000) showed that ACU stimulation directly decreases nitric oxide (NO) release, which is an important factor in hyperemia after ischemia (Greenberg *et al* 1995). The decreased NO release resulting from ACU stimulation contributes to neuroprotection during the reperfusion period. Therefore, reduced reperfusion %CBF, taken together with the present results, suggest the likelihood that ACU stimulation during the ischemic period suppresses hyperemia in the increased plasma osmolality (Duckrow 1995), and/or decreased levels of adenosine (Guyot *et al* 2001) under diabetic conditions, and that suppression leads to effects of a potential neuroprotection by ACU stimulation.

Changes in extracellular glutamate release

GLU is an excitatory neurotransmitter that plays an important role in the human brain. Excessive release of GLU is believed to be a cause of neuronal death associated with various neurological disorders. Therefore, extracellular levels of GLU, which may be neurotoxic to the brain, are considered a possible factor contributing to increased neural damage in diabetic ischemia (Farrokhnia *et al* 2005, Gilmore and Stead 2006, Ginsberg *et al* 1987, Kagansky *et al* 2001, Muranyi *et al* 2006). Interestingly, the ischemia-evoked increase in extracellular release of GLU due to DM might be associated with a greater excitatory amino acid burden with increased necrosis. However, the effects of DM on ischemia continue to be a controversial issue, where DM might reduce or increase extracellular GLU concentration.

Our previous study (Yin *et al* 2008a) also showed that the two groups, normal rats with and without ACU stimulation, showed a significant difference in extracellular ΔGLU, while the reduction of %CBF and reperfusion hyperemia were not significantly different between the two groups. Results were consistent with those reported previously in that stroke and might be associated with a decrease in ischemic/reperfusion ΔGLU by ACU stimulation (Pang *et al* 2003). Suppression of ΔGLU could be an important mechanism explaining the effect of ACU on ischemic stroke. GLU excitotoxicity is a major mechanism of neuronal cell death in diabetic rats under ischemic conditions, and is a probable target of therapeutic intervention (Gardoni and Di Luca 2006). Therefore, these results suggest that ischemic ACU stimulation

contributes to potentially the neuroprotective effect, saving neurons from acute and/or delayed neuronal cell death induced by ischemia in diabetic rats (Pang *et al* 2003).

Acupuncture stimulation

The selection of ACU point and method of stimulation are known to have different physiological effects. For example, Kim *et al* (2008) showed that during electro-ACU stimulation, a 2 Hz low frequency is more efficient than a 120 Hz high frequency. ACU points are generally selected by certified oriental medical doctors referring to the major classic ACU textbook (Koh and Yin 2007). ACU points GB34 and GB39 are traditionally known to belong to the category of eight meeting points and to demonstrate prominent therapeutic effects on motor function disorders in bone marrow and tendon (WHO 2007). These points are located on the lateral aspect of both rat hindpaws (figure 3), according to anatomical correspondence to human ACU points (Yin *et al* 2008b). This study employed a manual manipulation method that has frequently been applied in clinical settings. Further study will be necessary to observe the effects of diverse ACU points and ACU stimulation methods.

Conclusions

This study has demonstrated the neuroprotective effects of ACU stimulation under DM conditions. A 10 min period of bilateral occlusion was produced in the animals, and the ischemic period was followed by a 40 min reperfusion. A reproducible 11-VO ischemia model was induced as a global ischemia. Patterns of change in %CBF and EEG during ischemia/reperfusion indicate that the surgical setup and 11-VO model are suitable for monitoring the effect of ACU under conditions of diabetic ischemia. The real-time monitoring system used in our laboratory highlights new possibilities for a detailed analysis of the *in vivo* dynamics of changes in the neurotransmitter GLU after ischemia. We proposed 23 diagnostic parameters for a detailed analysis of changes in %CBF and GLU release during ischemia/reperfusion. We found that a significant decrease ($p < 0.05$) in the maximum responses to ischemic and reperfusion %CBF was observed in ACU rats. Compared with control rats, a significantly larger increase ($p < 0.05$) in the time elapsed to reach maximum %CBF levels during reperfusion was observed in ACU-treated DM rats. Furthermore, a significant decrease ($p < 0.05$) in maximum levels of ischemic and reperfusion ΔGLU was observed in ACU-treated DM rats. Compared with control rats, a significant decrease ($p < 0.05$) in the increment slope during the ischemic period, and increment and decrement slopes during the reperfusion period was observed in ACU-treated DM rats. We conclude that the decrease in %CBF and ΔGLU during diabetic ischemia is responsible for the potential neuroprotective effects of ACU stimulation in a rat model.

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